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Qualitative and Quantitative Analysis of β-carotene Using UPC²

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APPLICATION BENEFITS

- A fast UPC²™ method to separate the three most common carotenoids that minimizes the risk of degradation.
- The UPC² method is four times faster than the traditional methods of analysis, thereby, reducing organic solvent consumption by 85%.
- For the trageted analysis, the β-carotene extract in MTBE can be directly injected onto an ACQUITY UPC²™ System for analysis without the need for time-consuming evaporation and reconstitution steps.

WATERS SOLUTIONS

ACQUITY UPC² System with a photodiode array (PDA) detector

MassLynx® Software

ACQUITY UPC² HSS C_{18} SB Column

KEY WORDS

Carotenoids, lutein, β-carotene, lycopene, fat-soluble, vitamins, convergence chromatography, UPC²

INTRODUCTION

Carotenoids are natural pigments synthesized by plants and some microorganisms. For animals and humans, carotenoids play an important role in vision. Carotenoids also act as important antioxidants with a preventative effect for various diseases.[1-2] Since carotenoids cannot be synthesized *de novo* in the human body, humans need to acquire them through diet and supplements. In 2010, the market value of commercially used carotenoids was estimated to be $1.2 billion and projected to grow to $1.4 billion by 2018.[3] As more stringent legislation for regulatory compliance of micronutrients in fortified food products and dietary supplements is being enacted or contemplated, there is an increasing demand for rapid and reliable analytical methods for the analysis and quantification of carotenoids in a variety of matrices.[4] The speed of analysis is of particular importance because regulatory compliance monitoring often requires a large number of assays. In addition, many carotenoids are thermal- or photo-sensitive and highly susceptible to isomerization and chemical degradation. Prolonged analysis time could lead to inaccurate quantification results.

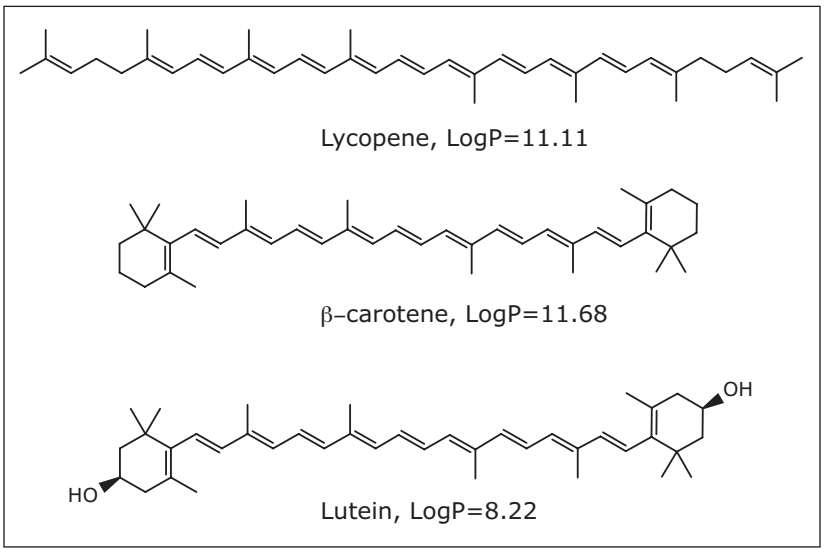

Figure 1. Chemical structures and LogP values of three carotenoids used in this study.

EXPERIMENTAL

UPC² conditions for column screening

System:	ACQUITY UPC²
Detection:	PDA detector
Flow rate:	1.5 mL/min
Mobile phase A:	CO_2
Mobile phase B:	Ethanol
Column:	ACQUITY UPC² BEH, CSH™ Fluoro-Phenyl, BEH 2-EP (3.0 x 100 mm, 1.7 µm), and HSS C_{18} SB (3.0 x 100 mm, 1.8 µm)
Back pressure:	2190 psi
SM temp.:	5 °C
Temp.:	40 °C
Sample diluent:	MTBE
Injection volume:	1 µL
Vials:	Waters® Amber Glass 12 x 32 mm Screw Neck Vial, 2 mL
PDA scan range:	220 to 600 nm
Data management:	MassLynx Software

Gradient:

Time (min)	B%
0	5
5	20
7	20
8	5
10	5

The central part of the carotenoid structure is the long polyene chain of alternating double and single bonds, as shown in Figure 1. Consequently, the carotenoids often possess high hydrophobicity, especially those that do not contain any hetero-atoms, such as lycopene and β-carotene. High-performance liquid chromatography (HPLC) with various absorbance detectors is the most commonly used analytical technique for determining carotenoids qualitatively and quantitatively.[1-2, 6-10] Due to their high hydrophobicity, separation of carotenoids by RPLC often results in lengthy analysis times. Furthermore, all RPLC-based methodologies generally suffer from the low solubility of carotenoids in the mobile phase. Non-aqueous reversed phase (NARP) LC has been employed to reduce the run time by using semi-aqueous or non-aqueous mobile phases. However, the NARP approach often involves the use of complex mixtures of organic solvents as the mobile phase. For example, in the official AOAC method for β-carotene in supplements and raw material,[11] a mixture of butylated hydroxytoluene (BHT), isopropanol, *N*-ethyldiisopropylamine, ammonium acetate, acetonitrile, and methanol is used as the mobile phase.

The separation of carotenoids has long been the subject of supercritical fluid chromatography (SFC)[12-18] studies since its inception.[12] The primary component of the mobile phase in SFC, CO_2, offers superior solubility for carotenoids and promotes non-polar interactions between carotenoids and the mobile phase, thereby reducing the retention time.[17] In addition to high chromatographic efficiency rendered by the high diffusivity of CO_2, the mild temperatures used in SFC are advantageous by avoiding thermal degradation of carotenoids.

UltraPerformance Convergence Chromatography™ (UPC²) is a new category of separation science that marries the merits of both SFC and UPLC. While adhering to the basic principles of SFC, UPC² leverages the reduced system volume of UPLC, and more importantly, the exceptional separation power of sub-2-µm particle packed columns, thereby, resulting in a greatly reduced run time, improved resolution, and increased detection sensitivity.

In this application note, we describe fast separations of three common carotenoids by UPC² in less than 2 minutes. A quantitative analysis of β-carotene dietary supplement capsules is also demonstrated.

EXPERIMENTAL

Optimized UPC^2 conditions for β-carotene extract analysis

Flow rate:	1.5 mL/min
Mobile phase:	75:25 CO_2/ethanol, isocratic
Column:	ACQUITY UPC^2 HSS C_{18} SB 3.0 x 100 mm, 1.8 µm
Back pressure:	2190 psi
SM temp.:	5 °C
Temp.:	40 °C
Sample diluent:	MTBE
Injection volume:	1 µL
Vials:	Waters Amber Glass 12 x 32 mm Screw Neck Vial, 2 mL
PDA scan range:	350 to 600 nm
Wavelength compensation:	440 nm with a reference wavelength 550 to 600 nm

Sample description

All sample preparation was performed in an environment with subdued lighting. For the column screening and subsequent optimization, 1 mg each of lycopene, β-carotene, and lutein was dissolved in 10 mL of methyl tert-butyl ether (MTBE) to make a 0.1 mg/mL (each) stock solution.

Calibration curve: A serial dilution of a stock solution of β-carotene (0.1 mg/mL in MTBE) was performed. The average peak area of three replicate injections at each concentration was used for each data point.

Capsule analysis: Three β-carotene capsules with a label claim of 15 mg/capsule were prepared by cutting them open and dissolving the contents in 250 mL of MTBE with slight perturbation. For each assay, six replicate injections were performed, and the average peak area was used for calculating β-carotene content in the capsules.

RESULTS AND DISCUSSION

Lutein, lycopene, and β-carotene are the three most common carotenoids found in the North American diet. Preliminary screening work revealed that methanol as mobile phase B (co-solvent) resulted in poor peak shape due to the low solubility of carotenoids in methanol, while isopropanol as a co-solvent led to broader peaks. Ethanol was, therefore, chosen as the co-solvent in all experiments. Figure 2 shows the UPC^2/UV chromatograms of the carotenoids mixture from the column screening. The peak identities were confirmed by injecting individual standard using the same condition. While the ACQUITY UPC^2 C_{18} SB Column yielded baseline resolution of all three carotenoids, another relatively non-polar column, CSH Fluoro-Phenyl, also provided partial separation between lycopene and β-carotene. No separation between lycopene and β-carotene was observed with either BEH or BEH 2-Ethyl Pyridine columns. Despite the similarities in structure and polarity between lycopene and β-carotene, the octadecyl carbon chains on the ACQUITY UPC^2 C_{18} SB stationary phase offered sufficient resolution to differentiate the two analytes in UPC^2.

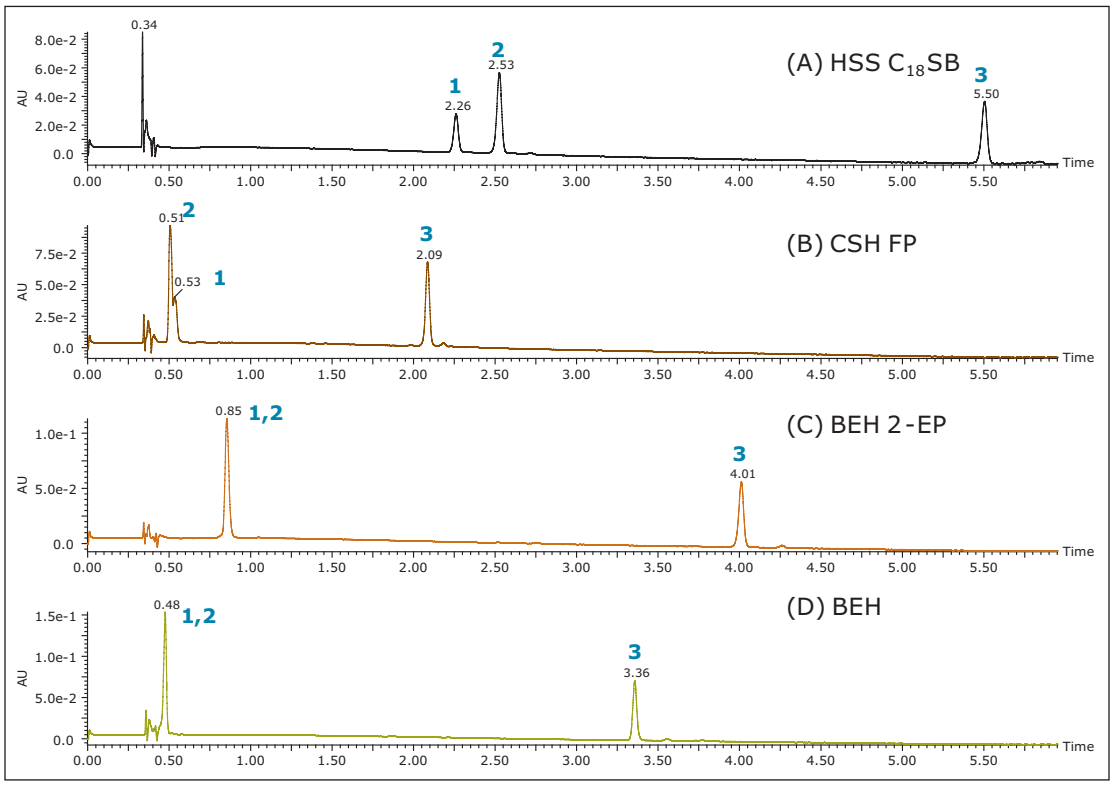

Figure 2. UPC^2/UV chromatograms of a mixture of lycopene, β-carotene, and lutein using the following different columns: (A) HSS C_{18} SB, (B) CSH Fluoro-Phenyl, (C) BEH 2-EP, and (D) BEH. The identities of the peaks are as follows: 1. Lycopene, 2. β-carotene, and 3. Lutein.

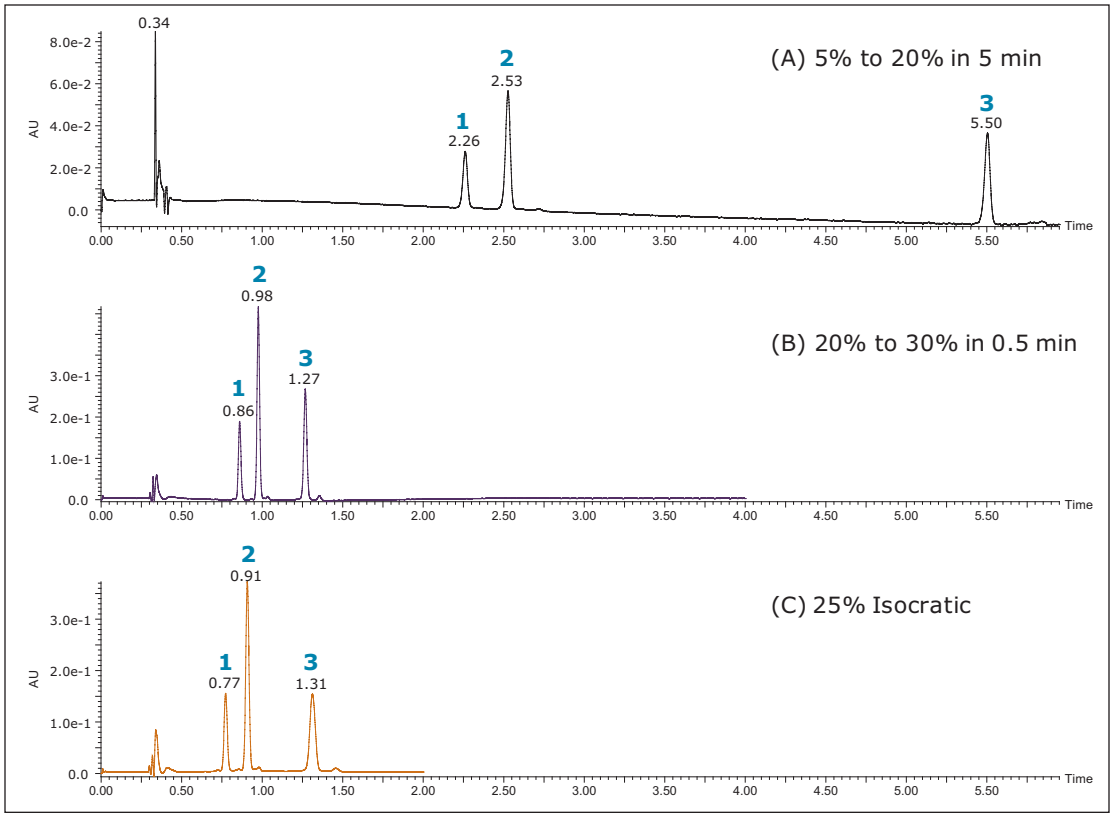

Figure 3. UPC²/UV chromatograms obtained using an ACQUITY UPC² HSS C_{18} SB Column under different gradient/isocratic conditions including: (A) the initial screening condition: 5% to 20% in 5 min, (B) 20% to 30% in 0.5 min, and (C) 25% isocratic. The identities of the peaks are: 1. Lycopene, 2. β-carotene, and 3. Lutein.

Next, an optimization step was performed to shorten the run time. A ballistic gradient of 20% B/min, shown in Figure 3B, and an isocratic method at 25% B, as seen in Figure 3C, both offered sufficient resolution for all three carotenoids with a run time of less than 2 min. The late-eluting peak (lutein) from the isocratic method has a slightly wider peak width than that from the gradient method, but the isocratic method generated a smoother baseline that can be beneficial for low level detection. The isocratic method was, therefore, chosen for ensuing quantitative analyses. The optimized method is four times faster than traditional methods of analysis.[5] As a result, the organic solvent consumption was reduced by ~85%. It is also important to note that for SFC using C_{18} columns, retention of non-polar analytes, such as carotenoids, decreases with the analytes' solubility in the mobile phase.[17] Since compressed CO_2 offers superior solubility for non-polar analytes, UPC² is inherently more compatible and faster for carotenoids analyses than RPLC.

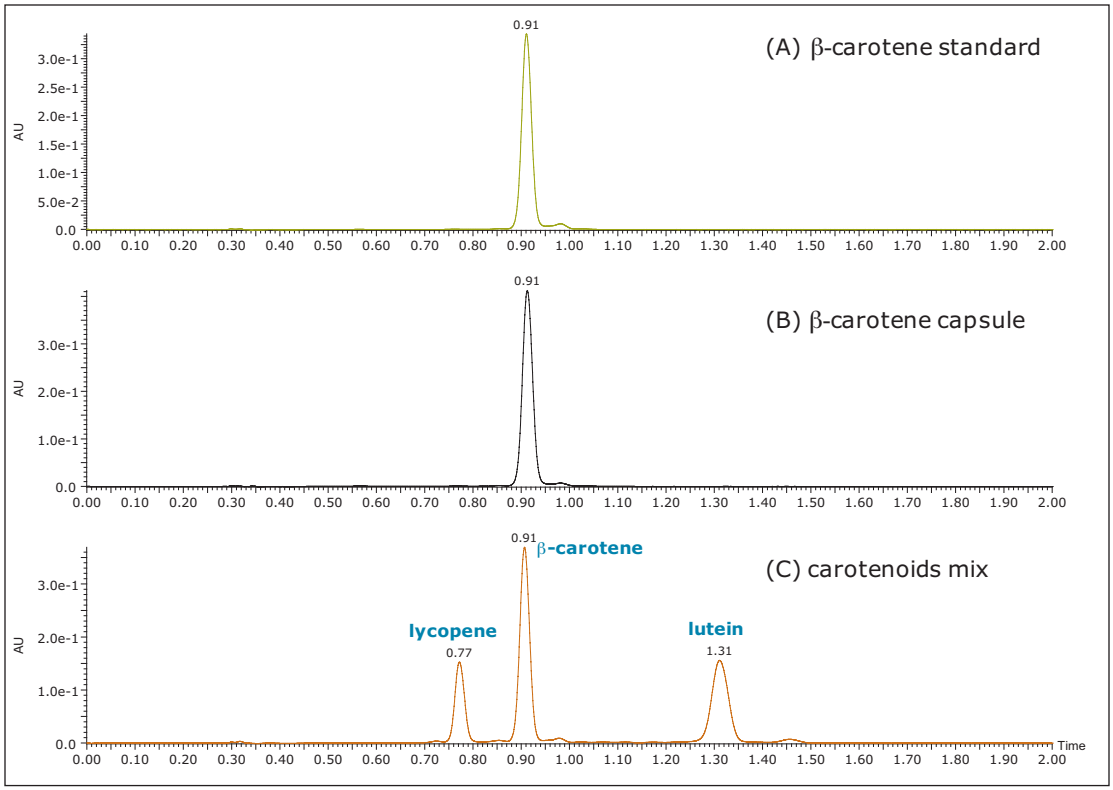

Figure 4. UPC² chromatograms of (A) β-carotene standard, (B) β-carotene extract from capsules, and (C) three carotenoids mixture under optimal chromatographic conditions.

For quantification, the β-carotene content of a commercially available capsule formulation was simply dissolved in MTBE, and the resulting extract was directly injected onto an ACQUITY UPC² System for analysis using the optimized method, shown in Figure 3C. A representative chromatogram of the resulting β-carotene extract is shown in Figure 4B. The simple sample preparation exemplifies another advantage of using UPC² for low polarity sample analysis. Dissolving low polarity samples often requires the use of low polarity solvents, such as MTBE and hexane, which are inherently compatible with UPC². In contrast, RPLC requires that samples dissolved in low polarity organic solvents be evaporated and reconstituted into suitable diluents prior to analysis.

Figure 5 shows a calibration curve for β-carotene in MTBE with concentrations ranging from 0.0001 to 0.1 mg/mL. The linearity range spans three orders of magnitude with $R^2 > 0.99$. The limit of detection (LOD, defined as S/N >3) and the limit of quantitation (LOQ, defined as S/N >10) are 50 and 100 ng/mL, respectively. These values are equivalent or better than those reported using HPLC.[6-8] The high detection sensitivity can be attributed to, in part, the inherent compatibility between carotene analysis and UPC^2. The non-polar interaction between CO_2 and β-carotene greatly reduces its retention, thus results in an early eluting sharp peak for improved detection sensitivity.

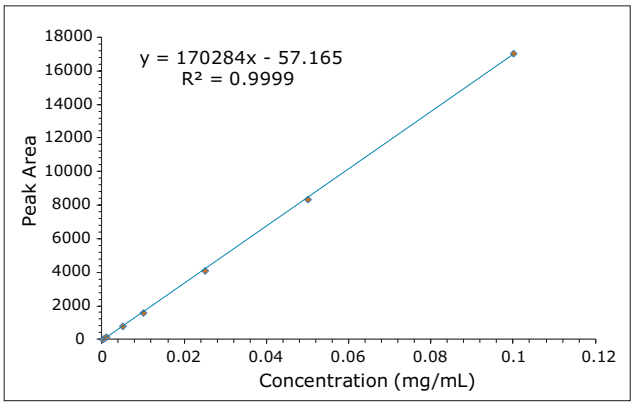

Figure 5. Calibration curve for β-carotene in MTBE with a concentration range of 0.0001 to 0.1 mg/mL in MTBE.

Tables 1 and 2 summarize the β-carotene capsule analyses. Excellent inter- and intra-assay reproducibility in both retention time and peak area was achieved. Overall assays also yielded good accuracy against the label claim. The sample preparation was simple and straightforward, and the chromatographic analyses using UPC^2 were fast and reproducible.

Injection	Peak area	Retention time (min)
1	10348	0.91
2	10291	0.91
3	10382	0.91
4	10330	0.91
5	10313	0.91
6	10293	0.91
Average	10326.17	0.91
RSD%	0.34	0

Table 1. Reproducibility of a β-carotene capsule assay with six replicate injections.

Label Claim: 15 mg/capsule				
Assay #1	Assay #2	Assay #3	Average	RSD%
mg/capsule				
15.13	15.39	15.24	15.25	0.84%

Table 2. Quantification of β-carotene in three capsules.

CONCLUSIONS

In summary, a UPC^2 method was successfully developed to separate the three most common carotenoids in less than two minutes. The method is four times faster than traditional methods of analysis, thereby reducing organic solvent consumption by 85%. The short analysis time also minimizes the risk of on-column degradation of the analytes. The improved speed of analysis is attributed to the inherent compatibility between UPC^2 and low polarity analytes. The UPC^2 method uses ethanol as the co-solvent instead of mixtures of organic solvents often used in HPLC methods. Thus, the UPC^2 method is a much more environmentally sustainable method.

A targeted 1.5-minute UPC^2 method was developed for the quantitative analysis of β-carotene in dietary supplement capsules. The dynamic range spans three orders of magnitude, with an LOD and an LOQ of 50 ng/mL and 100 ng/mL, respectively. Using MTBE as the extraction solvent, the resulting β-carotene extract can be directly injected onto an ACQUITY UPC^2 System for analysis without the need for time-consuming evaporation and reconstitution steps often associated with RPLC-based methodology. Excellent reproducibility and accuracy were also demonstrated for dietary supplement capsule analysis. The high-throughput UPC^2 method is ideally suited for laboratories routinely performing quality control and regulatory compliance monitoring where a large number of assays are required.

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